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The *Phycodnaviridae*: The Story of How Tiny Giants Rule the World

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The *Phycodnaviridae*: The Story of How Tiny Giants Rule the World

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Abstract

The family *Phycodnaviridae* encompasses a diverse and rapidly expanding collection of large icosahedral, dsDNA viruses that infect algae. These lytic and lysogenic viruses have genomes ranging from 160 to 560 kb. The family consists of six genera based initially on host range and supported by sequence comparisons. The family is monophyletic with branches for each genus, but the phycodnaviruses have evolutionary roots that connect them with several other families of large DNA viruses, referred to as the nucleocytoplasmic large DNA viruses (NCLDV). The phycodnaviruses have diverse genome structures, some with

large regions of noncoding sequence and others with regions of ssDNA. The genomes of members in three genera in the *Phycodnaviridae* have been sequenced. The genome analyses have revealed more than 1000 unique genes, with only 14 homologous genes in common among the three genera of phycodnaviruses sequenced to date. Thus, their gene diversity far exceeds the number of so-called core genes. Not much is known about the replication of these viruses, but the consequences of these infections on phytoplankton have global affects, including influencing geochemical cycling and weather patterns.

Introduction

The illuminated region is only a small part of the 3.7-km mean depth of the ocean, yet it houses several of the great engines of planetary control (Tett 1990). Absorption of heat energy by the ocean and light energy by tiny floating marine plants, known as phytoplankton, are the engines that help regulate many aspects of the global environment. Phytoplankton (microalgae) form the base of the marine food web and their photosynthetic activities provide an important carbon sink that influences the global carbon cycle and even climate (Charlson et al. 1987). Conservative estimates suggest there is somewhere between 100,000 to several million species of algae and that only approximately 40,000 have been identified. The *Phycodnaviridae* consists of a family of viruses that infect these globally important players. A literal translation of *Phycodnaviridae* is, DNA viruses that infect algae. Given the number of potential hosts, it is incredible so few phycodnaviruses have been isolated to date. With only approximately 150 formal identifications (Wilson et al. 2005b) and at least 100 others mentioned in the literature, it is certain that most phycodnaviruses, containing an almost infinite reservoir of genetic diversity, remain to be discovered.

The *Phycodnaviridae* comprise a genetically diverse (Dunigan et al. 2006), yet morphologically similar, family of large icosahedral viruses that infect marine or freshwater eukaryotic algae with dsDNA genomes ranging from 160 to 560 kb (Van Etten et al. 2002). Members of the *Phycodnaviridae* are currently grouped into six genera (named after the hosts they infect): *Chlorovirus*, *Coccolithovirus*, *Prasinovirus*, *Prymnesiovirus*, *Phaeovirus*, and *Raphidovirus* (Wilson et al. 2005b). Nomenclature anomalies have already crept into the system with the formation of the genus *Coccolithovirus*, a group of viruses that infect *Emiliania huxleyi* (an alga species in the class *Prymnesiophyceae*), which logically should have been classified within the genus *Prymnesiovirus*. However, phylogenetic analysis of the DNA polymerase gene from these viruses indicated they belong to a new genus (Schroeder et al. 2002). It is likely the *Phycodnaviridae* will eventually be split into several subfamilies and numerous genera as more isolates are characterized and the current nomenclature will probably become obsolete. Complete genome sequences have been obtained from representatives of the *Chlorovirus*, *Coccolithovirus* and *Phaeovirus* genera (Dunigan et al. 2006) and evolutionary analysis of their

genomes places them within a major, monophyletic assemblage of large eukaryotic dsDNA viruses termed the Nucleo-Cytoplasmic Large DNA Viruses (NCLDVs) (Iyer et al. 2001, 2006; Allen et al. 2006c; Raoult et al. 2004). There are five families in the NCLDV clade that include *Poxviridae*, *Iridoviridae*, *Asfarviridae*, *Phycodnaviridae* and *Mimiviridae*. Probably, the virus family *Ascoviridae* should also be included in this grouping because these viruses have clearly evolved from the Iridoviruses (see the chapter by V.G. Chinchar et al., this volume). The grouping of the NCLDVs is significant for two reasons: (i) as the name suggests, it implies a likely propagation mechanism for members of the *Phycodnaviridae* where replication is probably initiated in the nucleus and is completed in the cytoplasm; (ii) the NCLDVs are proposed to have an ancient evolutionary lineage (e.g., Villarreal and DeFilippis 2000; Iyer et al. 2006; Raoult et al. 2004). Phylogeny of the NCLDVs constructed by cladistic analysis indicates that the major families may have diverged prior to the divergence of the major eukaryotic lineages 2-3 billion years ago (Iyer et al. 2006; Raoult et al. 2004). The finding that there are only 14 genes in common, from a pool of approximately 1000 genes, between three genomes from different genera of the *Phycodnaviridae* (Allen et al. 2006c) supports the idea that the *Chlorovirus*, *Coccolithovirus* and *Phaeovirus* genera diverged a long time ago. As more *Phycodnaviridae* genomes are sequenced, there is likely to be an explosion of exciting gene discoveries with novel functions.

Reports of virus-like particles (VLPs) in at least 44 taxa of eukaryotic algae have appeared since the early 1970s (Van Etten et al. 1991). These include incidental observations of VLPs in electron micrographs, for example the first description of VLPs in *E. huxleyi* was reported in 1974 (Manton and Leadbeater 1974). Although these investigators did not publish an electron micrograph showing VLPs in *E. huxleyi*, they mentioned that VLPs in *Chrysochromulina mantoniae* (plates 65 and 66, Manton and Leadbeater 1974) resembled those of VLPs commonly found in moribund or dead *Coccolithus huxleyi* cells (now referred to as *E. huxleyi*). Many images containing VLPs are probably labeled miscellaneous and filed into obscurity in laboratories around the world (e.g., Figure 1). These old images could be a valuable resource to help identify new viruses and susceptible host strains. It was not until 1979 that a phycodnavirus was even isolated; the virus infected the marine unicellular alga *Micromonas pusilla* (Mayer and Taylor 1979). However, this report was largely ignored until the early 1990s (Cottrell and Suttle 1991) when high concentrations of viruses in aquatic environments were being described (Bergh et al. 1989). Perhaps most significantly, in the early 1980s a group of viruses were characterized that infect freshwater unicellular, eukaryotic, ex-symbiotic chlorella-like green algae, called chloroviruses (Meints et al. 1981; Van Etten et al. 1981, 1982, 1983a). These reports were followed in the early 1990s by research into marine filamentous brown algal viruses (Muller et al. 1990; Henry and Meints 1992; Muller and Stache 1992). Thereafter, use of genetic markers such as virus-encoded DNA polymerases, a core gene present in all NCLDVs (Iyer et al. 2001, 2006; Allen et al. 2006c), revealed that phycoviruses are a diverse and ubiquitous component of aquatic environments (Chen and Suttle 1995; Chen et al. 1996; Short and Suttle 2002). The field of

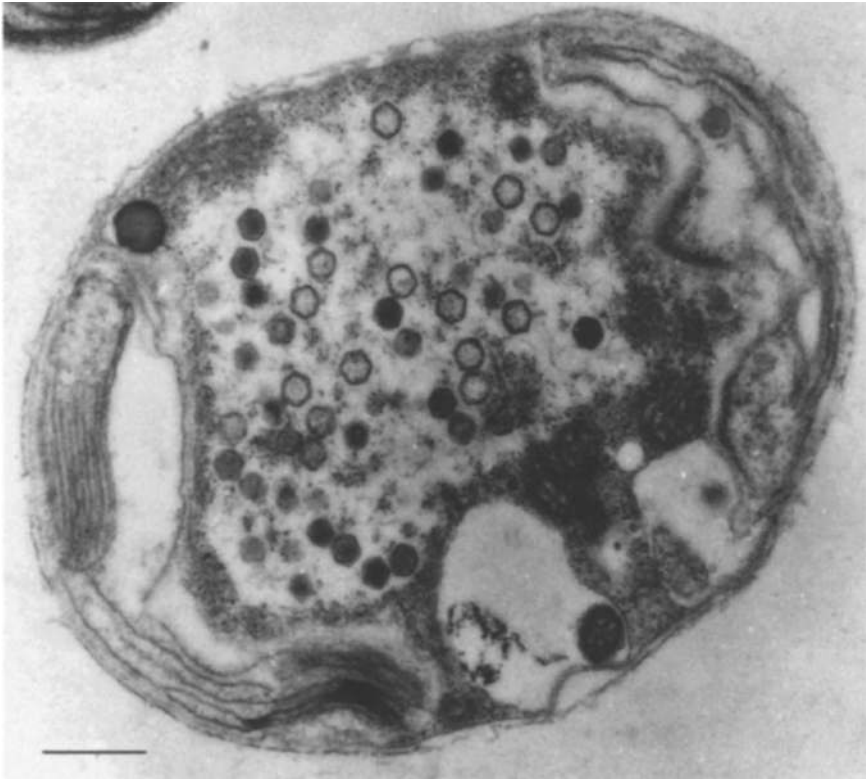


Figure 1. Final stages of infection in the marine phytoplankton *Pavlova virescens*. Note the different stages of virus assembly in the cell cytoplasm. Although never characterized, these VLPs have the hallmarks of a phycodnavirus. Samples were prepared by thin-sectioning in 1978! Image was given to the author when the investigator, John Green (formerly Marine Biological Association, Plymouth), was clearing out his office prior to retirement. No information was available on the size of the scale bar.

phycodnavirology is now well established and expanding rapidly; indeed there have been four international meetings dedicated to the subject (Bergen, Norway 1998; Galway, Ireland 2000; Hiroshima, Japan 2002; and Amsterdam, Netherlands 2005). Previous algal virus reviews have focused primarily on the chloroviruses (Van Etten and Meints 1999; Van Etten et al. 2002, 2003; Kang et al. 2005; Dunigan et al. 2006; Yamada et al. 2006) because of the extensive research on these viruses. The ecological aspects of marine phytoplankton viruses were reviewed recently by Brussaard (2004). The current chapter will provide a broad overview of the *Phycodnaviridae*, attempting to describe the novelty and incredible genetic diversity of this ancient group of viruses. Starting with a brief introduction to the algal hosts these viruses infect, the review will then cover virus propagation strategies, genome structure, an analysis of known and novel genes, and finish with a discussion on core genes and their implication in *Phycodnaviridae* evolution.

Hosts

Eukaryotic algae are a group of oxygen-evolving, photosynthetic organisms that include seaweeds (macroalgae) and a large diverse group of microorganisms generically referred to as microalgae. Photosynthetic prokaryotes such as pico-cyanobacteria (e.g. *Synechococcus* or *Prochlorococcus*) are not included in this group, however, they are infected by bacteriophage-like viruses (Suttle 2000a, 2000b; Clokie and Mann 2006). Eukaryotic algae range in size from the smallest known eukaryote, *Ostreococcus*, at approximately 1 μm in diameter (Derelle et al. 2006; Palenik et al. 2007), through numerous chain-forming and colonial species that are visible to the naked eye to the large kelp (seaweed) forests in coastal regions. Algae consist of at least five distinct evolutionary lineages (plants, cercozoa, alveolates, heterokonts and discicristates) (Baldauf 2003) and they are ubiquitous in marine, freshwater and terrestrial habitats. The number of algal species (mostly microalgae) has been estimated to be as high as several million; hence their overall diversity is probably enormous. It is likely that viruses infect all of these species; furthermore, not all of these viruses will be assigned to the *Phycodnaviridae* family. Indeed, other types of viruses that infect algae are being discovered and characterized (e.g., ssRNA, dsRNA, and ssDNA containing viruses, Tai et al. 2003; Brussaard et al. 2004a; Nagasaki et al. 2004). Thus, algal virology is in its infancy.

Chlorella and *Chloroviruses*

The genus *Chlorella* consists of small, unicellular, non-motile, asexual green algae with a global distribution (Figure 2A) (e.g., Shihra and Krauss 1965). They have a simple developmental cycle and reproduce by mitotic division. Vegetative cells increase in size and, depending on the species and environmental conditions, divide into two, four, eight or more progeny, which are released by rupture or enzymatic digestion of the parental walls. *Chlorella* species are usually free living, although many species have symbiotic relationships with organisms from different classes in the animal kingdom including *Rhizopoda*, *Ciliata*, *Hydrozoa* and *Turbellaria* (Reisser 1992). To date, the only described chloroviruses infect symbiotic *Chlorella*, often referred to as zoochlorellae, such as those associated with the protozoan *Paramecium bursaria*, the coelenterate *Hydrozoa viridis* and the heliozoon *Ancanthocystis turfacea* (Kawakami and Kawakami 1978; Meints et al. 1981; Van Etten et al. 1982; Bubeck and Pfitzner 2005). Fortunately, many of these *Chlorella* strains can be grown in the laboratory, independent of their symbiotic partner, including *P. bursaria*-associated *Chlorella* isolates NC64A and Pbi. The chloroviruses can be produced in large quantities and assayed by plaque formation. Chloroviruses are ubiquitous in nature and have been isolated from freshwater collected throughout the world (Yamata et al. 2006). Typically, the virus titers in native waters are 1-100 plaque-forming units (PFU) per ml, but titers as

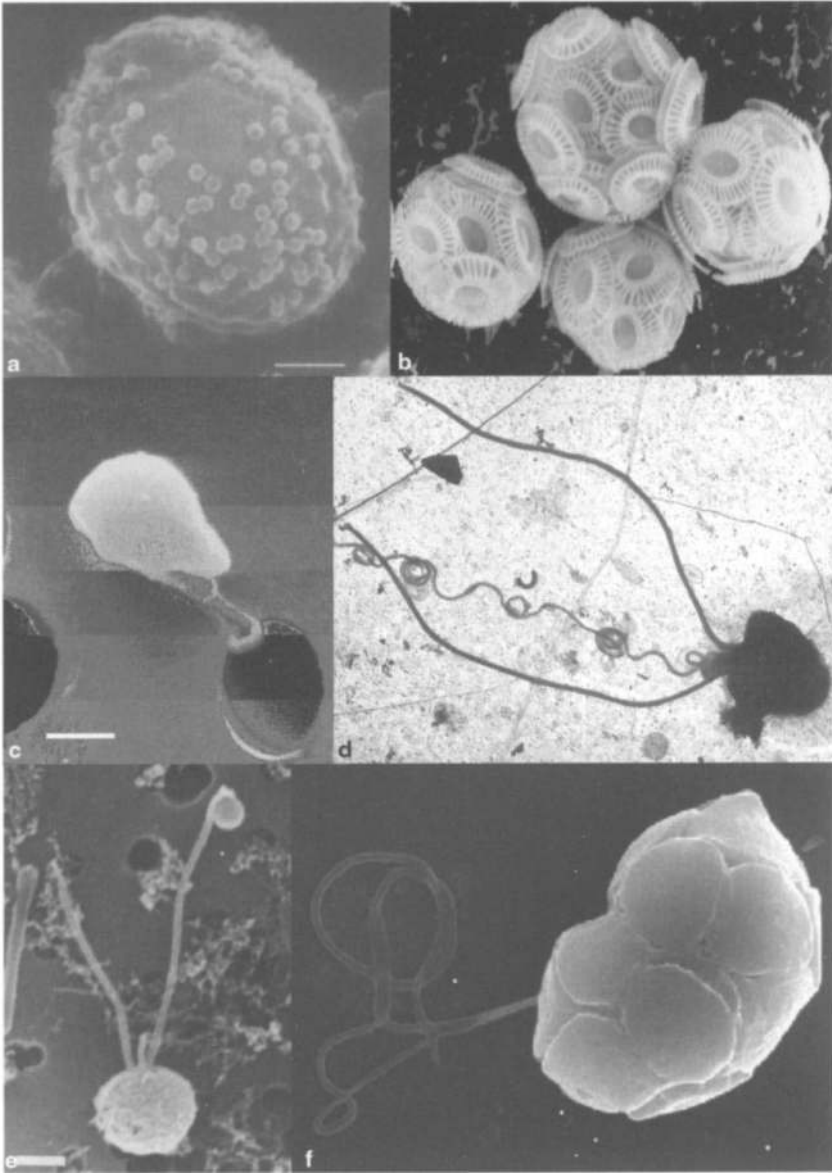


Figure 2. Pictures of some representative algae that are hosts for phycodnaviruses. **a)** Scanning electron micrograph (SEM) of *Chlorella* NC64A with PBCV-1 particles attached to its surface (scale bar, approx. 500 nm), **b)** SEM of four *Emiliana huxleyi* cells (each cell, approx. 5 μm in diameter), **c)** SEM *Micromonas pusilla* (scale bar, approx. 500 nm). **d)** Transmission electron micrograph (TEM) of a *Chrysochromulina* sp. cell in a seawater sample taken from a Norwegian fjord (cell diameter, approx. 3 μm). **e)** SEM of *Phaeocystis* sp. (strain Naples) (scale bar, approx. 1 μm). **f)** SEM of *Heterosigma akashiwo* (cell diameter, approx. 12 μm). Images courtesy of (a) J.L. Van Etten (Meints et al. 1984); (b) W. Wilson (unpublished); (c) Bengt Karlson (unpublished) (d) G. Bratbak and M. Heldal (unpublished); (e) D. Vaultot (Vaultot et al. 1994); (f) S. Itakura and K. Nagasaki (unpublished).

high as 100,000 PFU/ml of native water have been obtained. Titers fluctuate with the seasons, with the highest titers occurring in the spring. Chlorovirus PBCV-1, which infects *Chlorella* NC64A, is currently the best-studied phycodnavirus (Van Etten 2003; Yamada et al. 2006).

Emiliania huxleyi and *Coccolithoviruses*

Emiliania huxleyi (Lohmann) Hay et Mohler is a representative of the coccolithophores, a group of unicellular calcifying marine microalgae that have a global distribution (Brown and Yoder 1994). In open oceanic environments, they constitute a significant fraction of the phytoplankton and have an exceptionally rich fossil record spanning approximately 200 million years (Bown 1998), with *E. huxleyi* appearing approximately 260,000 years ago. *E. huxleyi* is an important species with respect to past and present marine primary productivity, sediment formation and climate change. It is the most numerous coccolithophore in our oceans and satellite observations of mesoscale *E. huxleyi* blooms (Holligan et al. 1983, 1993) verified what marine geologists had long known, that calcite derived from these vast blooms is an important component of marine carbon. Ironically, the elaborate calcium carbonate armory of liths covering the surface of these cells was always thought to prevent virus infection (Figure 2B).

E. huxleyi greatly impacts marine ecosystems and, in particular, the global carbon and sulphur cycles (Westbroek et al. 1993; Burkill et al. 2002). Blooms of this ubiquitous microalga are known to affect the oceanic carbon pump (Elderfield 2002) and climate (Charlson et al. 1987). Vast coastal and mid-ocean populations of *E. huxleyi* often suddenly disappear, causing substantial fluxes of calcite to the seabed (Ziveri et al. 2000) and cloud-forming dimethyl sulfide to the atmosphere (Malin 1997; Evans et al. 2007). Until recently, the mechanisms of *E. huxleyi* bloom disintegration were poorly understood but it is now commonly accepted that viruses are intrinsically linked to these sudden crashes (Bratbak et al. 1993, 1996; Brussaard et al. 1996; Castberg et al. 2002; Jacquet et al. 2002; Wilson et al. 2002; Schroeder et al. 2003).

Prasinophytes and *Prasinoviruses*

Prasinophytes belong to a group of marine phytoplankton, generically referred to as photosynthetic picoeukaryotes (PPEs), which consist of small (>3 μm) photosynthetic eukaryotic cells. They are a ubiquitous, abundant and highly diverse component of the world's oceans. This abundance, coupled with their high carbon fixation rates (Li 1994; Worden et al. 2004), means that PPEs are important contributors to biomass production in the open ocean, despite usually being numerically outnumbered by photosynthetic prokaryotes (e.g., *Synechococcus* or *Prochlorococcus*). The incredibly high diversity of PPEs is

only now being appreciated (Diez et al. 2001; Moon-van der Staay et al. 2001; Zeidner et al. 2003; Fuller et al. 2006; Worden 2006). Among the many classes included in the PPEs, members of the class Prasinophyceae are ubiquitous in clone libraries collected from oceanic samples and they often dominate the PPE community (Not et al. 2004; Worden 2006). These include members in the genera *Ostreococcus*, the smallest known eukaryote (Chrétiennot-Dinet et al. 1995), *Bathycoccus* (Eikrem and Thronsen 1990), and *Micromonas* (Figure 2C), a major component in many oceanic and coastal regions (Not et al. 2005).

Despite the obvious ubiquity and abundance of these algae, relatively little research has been conducted on prasinoviruses and only a few viruses have been described. This is perhaps ironic, given that the first characterized virus assigned to the *Phycodnaviridae* was a prasinovirus that infects *Micromonas pusilla* (Mayer and Taylor 1979). *M. pusilla*-specific viruses are the only prasinoviruses that have been described. These viruses are easy to isolate, simply adding a small volume of filtered coastal seawater to an exponentially growing culture of *M. pusilla* usually results in virus-induced lysis. Research has focused largely on their ecological role and their genetic diversity (Cottrell and Suttle 1991, 1995b; Sahlsten 1998; Sahlsten and Karlson 1998; Zingone et al. 1999, 2006). *M. pusilla* viruses can lyse up to 25% of the daily *Micromonas* population (Evans et al. 2003); however, the high growth rates of the hosts, coupled with the high diversity of both host (Worden 2006) and virus (Chen et al. 1996), allows them to propagate in a stable co-existence (Cottrell and Suttle 1995a) as compared to the bloom-bust scenario observed with *Coccolithovirus* infection of *E. huxleyi* blooms (Bratbak et al. 1993; Jacquet et al. 2002; Wilson et al. 2002).

Except for a report of a transient *Ostreococcus* bloom off the eastern coast of the United States, whose rapid decline was partially attributed to viruses (O'Kelly et al. 2003), no other reports exist of viruses infecting prasinophytes. However, the authors are aware of an unpublished investigation on the isolation of prasinoviruses that infect the smallest known eukaryotic cell, *Ostreococcus tauri*. These dsDNA viruses are readily isolated by plaque assay and are morphologically similar to the *M. pusilla* viruses (K. Weynberg, N. Grimsley, H. Moreau, personal communication). Of the six *Phycodnaviridae* genera, the prasinoviruses and viruses of PPEs are the least represented in the literature despite their global and ecological importance.

Prymnesiophytes and Prymnesioviruses

Algae assigned to the class Prymnesiophyceae, from the division Haptophyta (and generally referred to as haptophytes), occur predominantly in marine environments. These algae have a global distribution and they are often associated with large-scale microalgal blooms. The principle characteristic of the haptophytes is the haptonema, a filiform organelle or Hagella whose length ranges from barely detectable (Green and Pienaar 1977) to many times that of the mother cell (Figure 2D) (Parke et al. 1959). The presence of two-layered microfibrillar scales on the cell surface is also a characteristic and it is traditionally considered an important taxonomic character at the species

level. In addition, the scales allow easy identification in mixed natural assemblages of microalgae (Leadbeater 1972). To date, prymnesioviruses have been isolated from members of the genera *Chrysochromulina* (Suttle and Chan 1995; Sandaa et al. 2001) and *Phaeocystis* (Jacobsen et al. 1996; Brussaard et al. 2004b; Wilson et al. 2006). One of the earliest reports of a phycodnavirus was that of a putative prymnesiovirus observed in thin sections of a *Chrysochromulina* sp. (Manton and Leadbeater 1974).

Chrysochromulina (Figure 2D) is considered a cosmopolitan genus; however, information on the distribution and abundance of *Chrysochromulina* at the species level is limited. Although abundance over an annual cycle is highly variable and generally low, high N:P ratios, stratified conditions with a low salinity surface layer during summer, together with low phosphate concentrations favor growth of *Chrysochromulina* spp. in coastal waters (Dahl et al. 2005). On the rare occasions when they do bloom, they can produce toxins that cause catastrophic damage to fisheries: for example, in 1988 a large *C. polylepis* bloom (~100,000/ml) off southern Norway killed large numbers of both wild and caged fish (Dundas et al. 1989). However, large monospecific blooms of *Chrysochromulina* spp. are rare; typically they are present in low concentrations (Estep and MacIntyre 1989). This property has led to speculation that *Chrysochromulina* spp. infecting viruses only require a low host density for propagation (Sandaa et al. 2001) and that the viruses may even prevent bloom formation (Suttle and Chan 1995).

In contrast, a lot of information exists on the biogeochemical impact on the marine ecosystem by members of the genus *Phaeocystis* (Figure 2E); these interdisciplinary studies are summarized in several recent reviews (Veldhuis and Wassmann 2005; Verity et al. 2007). *Phaeocystis* spp. form dense spatially and temporally extensive monospecific blooms consisting of a mixture of colonial (within a gelatinous matrix) and unicellular cells that collapse suddenly in a virus-induced crash (Brussaard et al. 2005a). This crash leads to a rapid shift in the composition of the bacterial community due to the massive flux of released organic nutrients (Brussaard et al. 2005b). Intense *Phaeocystis* spp. blooms can lead to anoxia and impressive foam formation on beaches during their decline (Lancelot et al. 1987), hence their label as harmful algal blooms (HABs). Similar to *E. huxleyi*, *Phaeocystis* spp. play important roles in CO₂ and sulphur cycling, which ultimately have major implications in the global climate² (Liss et al. 1994) and infection by viruses can exacerbate this process (Malin et al. 1998).

Phaeophyceae and Phaeoviruses

The class Phaeophyceae is more commonly referred to as the brown algae. They are an important economic resource that includes uses in the cosmetic, food and fertilizer industries as well as a source of important biomolecules (McHugh 1991). As multicellular, filamentous macroalgae, they differ from the other unicellular, microalgal hosts of the *Phycodnaviridae*. Another major difference is that during infection, the virus genomes are integrated into

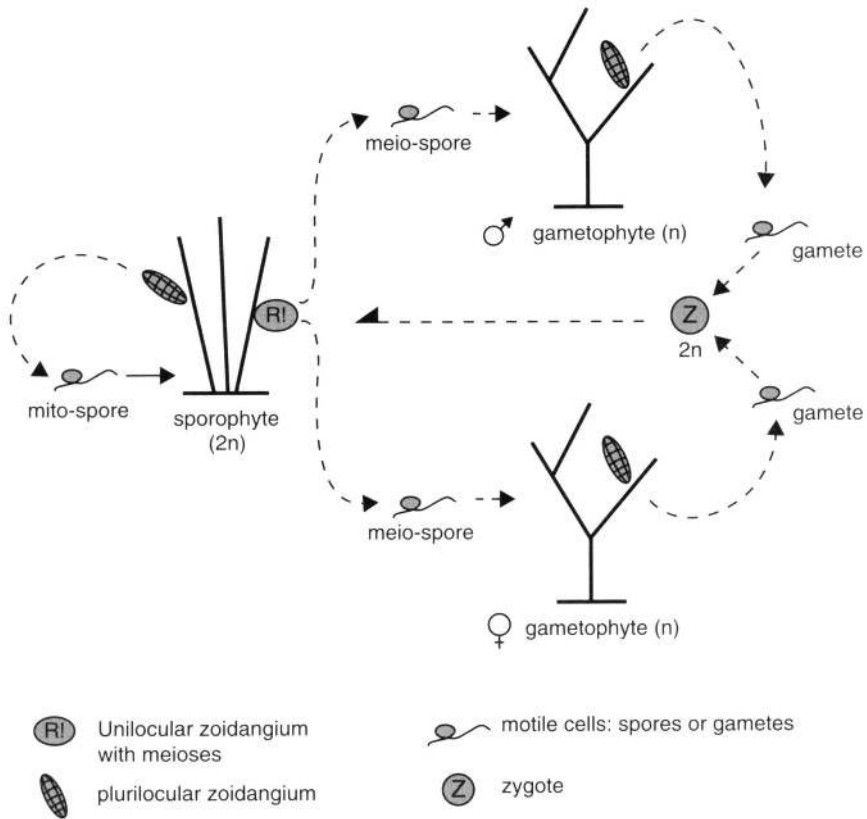


Figure 3. Life cycle of *Ectocarpus siliculosus*. Zoidangium: sporangium on sporophytes, gametangium on gametophytes. Note that the sporophyte possesses two types of reproductive organs: (1) unilocular sporangia where meiosis occur (R^1) producing haploid (n) meiospores, and (2) plurilocular sporangia where diploid ($2n$) mitospores are produced by multiple mitoses. (Reproduced with permission from Müller DG et al. 1998).

the host genome; the virus genomes are then inherited in a Mendelian fashion and virus particles only appear in the reproductive organs of the infected algae (Müller et al. 1998). Phaeophyceae pre-dominantly inhabit temperate near-shore marine coastal environments around the world. They can be significant components of fouling communities that occur on marine structures such as docks, buoys and the exterior surfaces of ships. Viruses are known to infect members in the genera *Ectocarpus*, *Feldmannia*, *Hincksia*, *Myriotrichia*, and *Pilayella*. The life cycle of the filamentous brown alga, *Ectocarpus* (Figure 3) is perhaps the best studied and it serves as a model organism for studying the molecular genetics of brown algae (Peters et al. 2004). *Ectocarpus* has a complicated sexual life cycle comprising alternating generations. Male and female gametophytes carry plurilocular zoidangia, which produce swimming gametes. The viruses only infect these free-swimming gametes. Gam-

ete fusion results in zygotes that develop into diploid sporophytes. The sporophytes produce unilocular sporangia where meiosis leads to the formation of haploid spores. These spores go on to produce the next gametophyte generation. The sporophytes can also develop plurilocular sporangia by mitotic cell division. The diploid spores can therefore repeat the diploid sporophyte phase (Müller et al. 1998). Virus particles are only formed in gametangia or sporangia (zoidangia) cells of the host (Müller 1996). Since viruses displace the normal reproductive organs, the algae are rendered sterile; however, this does not affect their vegetative growth (Müller et al. 1990).

Raphidophytes and Raphidoviruses

Microalgae in the class Raphidophyceae are important bloom-forming species found in coastal and subarctic regions of the world's oceans, although freshwater species also exist (Figueroa and Rengefors 2006). Members are often associated with toxic red tides (Khan et al. 1997) and subsequent fish kills, particularly in the aquaculture industry (Hiroishi et al. 2005). Classical identification of the Raphidophytes relies on morphological differences, but it is extremely difficult to distinguish some of the species due to a pleomorphism that changes shapes and sizes in various environmental conditions (Khan et al. 1995). Consequently, efforts to develop rapid and sensitive polymerase chain reaction (PCR)-based diagnostic techniques are ongoing (Kai et al. 2006). To date, the only viruses reported in this genus infect the red tide forming species *Heterosigma akashiwo* (Figure 2F); the viruses are referred to as HaV (*Heterosigma akashiwo* virus) (Nagasaki and Yamaguchi 1997, 1998a; Nagasaki et al. 1999b). *H. akashiwo* is a single species belonging to the genus *Heterosigma* (Thronsdon 1996) and, although not usually associated with human illness, blooms of *H. akashiwo* have caused massive fish kills, typically of caged fish such as salmon and yellowtail (Honjo 1993; Khan et al. 1997). The susceptibility of *H. akashiwo* to HaV differs among clonal strains in the laboratory (Nagasaki and Yamaguchi 1998b; Nagasaki et al. 1999a; Tarutani et al. 2006). Marine field surveys and cross-reactivity tests between *H. akashiwo* host strains and HaV clones suggest that this strain-dependent infection plays an important role in determining clonal composition and effectively maintaining intraspecific diversity in natural *H. akashiwo* populations (Tarutani et al. 2000; Tomaru et al. 2004). This diversity probably contributes to the success of *H. akashiwo* as a ubiquitous and problematic bloom former in coastal regions.

Virion Morphology/Structure

Despite the wide host range displayed by the *Phycodnaviridae*, all members have similar structural morphology; this is consistent (independent of genomic information) with a common ancestry. Few have been studied in great detail, but the overlying pattern suggests a general conservation of structure. The life-

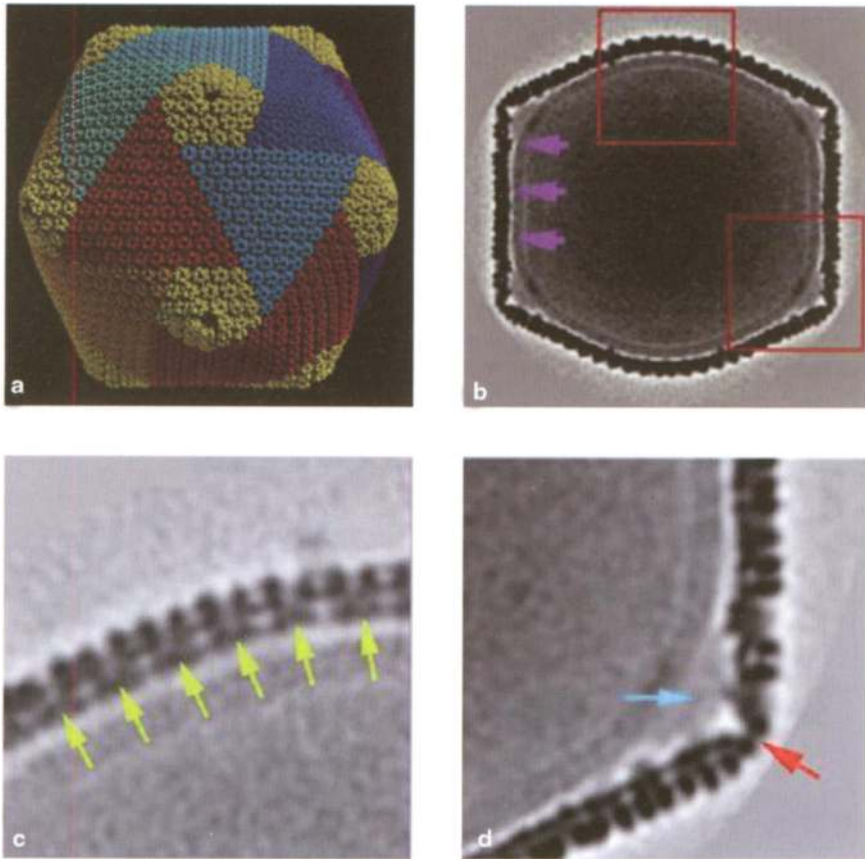


Figure 4. Three-dimensional image reconstruction of chlorella virus PBCV-1 from cryoelectron micrographs, **a)** The virion capsid consists of 12 pentasymmetrons (highlighted in *yellow*) and 20 trisymmetrons (highlighted in *red* and *two shades of blue*). A pentavalent capsomer lies at the center of each pentasymmetron. Each pentasymmetron consists of one pentamer plus 30 trimers. Eleven capsomers form the edge of each trisymmetron and therefore each trisymmetron has 66 trimers. **b)** Cross-sectional view of PBCV-1 along the twofold axis. A lipid bilayered membrane, like a railroad track, exists beneath the capsid shell (*magenta arrows*). Magnified views at two- and fivefold axes (outlines in **b** are shown in **c** and **d**, respectively), **c)** The capsomers are interconnected by “cement” proteins (*yellow arrows*), **d)** Dense material (*blue arrow*) (cell wall-digesting enzyme(s)?) is present at each vertex (*red arrow*) between the vertex and the membrane, (**a** reproduced with permission from Simpson et al. 2003. **b-d** reproduced with permission from Van Etten 2003)

style of viruses, i.e., they enter a cell, hijack the cell metabolic machinery and make hundreds to thousands of new viruses, creates a vital need for a simple and easily assembled virion particle, hence the conservation of structure (Bamford et al. 2002). The virions are large (to contain the large genomes) layered structures 100-220 nm in diameter with a dsDNA-protein core surrounded by a lipid bilayer and an icosahedral capsid (Figure 4) (Nandhagopal et al. 2002). The capsids have 20 equilateral triangle faces composed of protein subunits

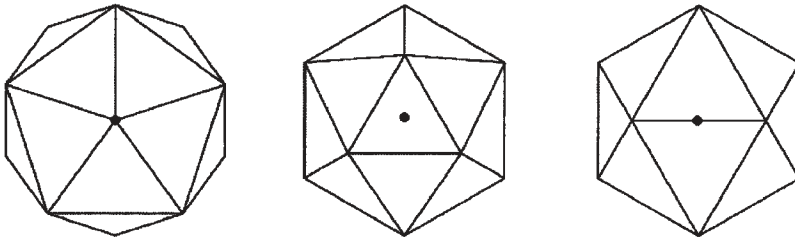


Figure 5. The *Phycodnaviridae* capsids have 20 equilateral triangle faces composed of protein subunits and are defined by having a two-, three- and fivefold axis of symmetry. From left to right, there are six fivefold axes of symmetry passing through the vertices, ten threefold axes extending through each face and 15 twofold axes passing through the edges of an icosahedron. (Diagram produced by Thomas Locke).

and are defined by having two-, three- and fivefold axis of symmetry (Crick and Watson 1956). There are six fivefold axes of symmetry passing through the vertices, ten threefold axes extending through each face and fifteen twofold axes passing through the edges (Figure 5). Like other icosahedron virions, the capsids are composed of well-ordered capsid substructures known as trisymmetrons (triangular) and pentasymmetrons (pentagonal) which are centered on the three- and fivefold axes of symmetry (Wrigley 1969; Stoltz 1971). The trisymmetrons do not represent the triangular faces of the icosahedron, but instead curve around the edges allowing the pentasymmetrons to fill the gaps at the fivefold vertices (Figure 4) (Nandhagopal et al. 2002; Simpson et al. 2003). In all of the *Phycodnaviridae* studied so far, the capsid is composed of 20 trisymmetrons and 12 pentasymmetrons (Yan et al. 2000, 2005). Trisymmetrons and pentasymmetrons are made up of trimeric capsomers, with each doughnut-shaped capsomer composed of three monomers of the major capsid protein.

Most of these data are based on PBCV-1. Cryoelectron microscopy and three-dimensional (3D) image reconstruction of the PBCV-1 virion at 26-Å resolution indicate that the outer capsid is icosahedral and covers a lipid bilayer membrane (Figure 4A, B) (Yan et al. 2000; Simpson et al. 2003). The membrane is required for infection, because PBCV-1 loses infectivity after exposure to organic solvents. The outer diameter of the viral capsid ranges from 1,650 Å along the two- and threefold axes to 1,900 Å along the fivefold axis. The capsid shell consists of 1,680 donut-shaped trimeric capsomers plus 12 pentameric capsomers at each icosahedral vertex. The trimeric capsomers are arranged into 20 triangular facets (trisymmetrons, each containing 66 trimers) and 12 pentagonal facets (pentasymmetrons, each containing 30 trimers and one pentamer at the icosahedral vertices). Assuming all the trimeric capsomers are identical, the outer capsid of the virus contains 5,040 copies of the major capsid protein, Vp54. PBCV-1 has a triangulation number (T number) of 169.

Most of the PBCV-1 trimeric capsomers have a central, concave depression surrounded by three protruding towers. The trimeric capsomers are 72 Å in diameter and 75 Å high. The prominent, cylinder portion of each trimer extends 50 Å above the surface of the capsid shell. The capsomers interconnect at their bases in a contiguous shell that is 20-25 Å thick. Within each tri-

mer, the three monomers connect in the middle; thus each trimer has the appearance of the letter H when viewed in cross-section. This structure agrees with the Vp54 structural studies (Nandhagopal et al. 2002). Cross-sectional views of the reconstructions reveal a “cement” protein(s) that connects the bases of the trimeric capsomers (arrow in Figure 4C).

Twelve pentamer capsomers, each 70 Å in diameter, exist at the virus fivefold vertices and probably consist of a different protein. Each pentamer is surrounded by radially distributed trimers, and the axis of each of these trimers tilts away from the pentamer. Each pentamer has a cone-shaped, axial channel at its base. One or more proteins appear below the axial channel and outside the inner membrane (Figure 4D). This protein(s) may be responsible for digesting the host cell wall during infection. Presumably contact between the virus and its host receptor alters the channel sufficiently to release the wall-degrading enzyme(s).

The PBCV-1 virion contains more than 110 different virus-encoded proteins (D.D. Dunigan et al., unpublished results). The PBCV-1 54-kDa major capsid protein (Vp54) is a glycoprotein and comprises approximately 40% of the total virus protein. Vp54 consists of two eight-stranded, antiparallel (β -barrel, jelly-roll domains related by pseudo-sixfold rotation (Nandhagopal et al. 2002). The glycan portion of Vp54, which is oriented to the outside of the particle, contains seven neutral sugars, glucose, fucose, rhamnose, galactose, mannose, xylose, and arabinose (Wang et al. 1993). The Vp54 crystal structure revealed six glycans attached to the protein, four N-linked glycans and two O-linked glycans (Nandhagopal et al. 2002). However, the four glycosylated Asn residues are not located in consensus sequences typical of eukaryotic N-linked glycans. This finding, together with other results (Wang et al. 1993; Que et al. 1994; Graves et al. 2001), led to the conclusion that, unlike other viruses, PBCV-1 encodes at least part, if not all, of the machinery required to glycosylate its major capsid protein (Markine-Goriaynoff et al. 2004). Furthermore, glycosylation probably occurs independently of the host endoplasmic reticulum-Golgi system.

The Vp54 structure resembles the major coat proteins from some other, smaller dsDNA viruses representing all three domains of life, including bacteriophage PRD1, human adenoviruses, and a virus infecting the Archaea, *Sulfolobus solfataricus*. This finding led to the suggestion that these three viruses also have a common evolutionary ancestor with the NCLDVs, even though there is no amino acid sequence similarity among their major capsid proteins (Bamford et al. 2002; Benson et al. 2004).

The Pymnesiovirus *Phaeocystis pouchetii* virus (PpVOI) has the largest capsid of the *Phycodnaviridae* and has a maximum outer diameter of 2200 Å between opposed vertices (fivefold axes), which drops to 1900 Å along the two- and three- fold axes (Yan et al. 2005). Like PBCV-1, the pentasymmetrons are composed of 31 capsomers (30 trimers and 1 pentamer); however, the trisymmetrons are larger, containing 91 trimers (13 per \sim 100 nm edge). PpVOI has a T number of 219.

The nature and origin of the lipid bilayer membrane remains relatively unstudied in the phycodnaviruses, although experiments in progress indicate that the PBCV-1 membrane probably originates from the endoplasmic reticulum (J.

Heuser et al., unpublished results). A lipid profile of virus EhV-86 is very similar to that of its host *E. huxleyi*, suggesting the direct requisition of host membrane lipids during virion assembly (T. Dunn, personal communication).

Over the last decade we have been increasingly aware that the *Phycodnaviridae* is a highly diverse family whose members share very little else in common other than the nature of their genomic material and their morphology. The following sections discuss this diversity with regards to lifestyle, genomic structure, coding and noncoding potential and infection strategy.

Propagation Strategies

Members of the *Phycodnaviridae* have a variety of lifestyles. The replication strategies of three of these viruses will be briefly described in this section, PBCV-1 representing the chloroviruses, EsV-1 representing the phaeoviruses and EhV-86 representing the coccolithoviruses.

PBCV-1 initiates infection by attaching rapidly, specifically and irreversibly to the chlorella cell wall (Meints et al. 1984, 1988), probably at a unique virus vertex (Onimatsu et al. 2006); attachment is immediately followed by degradation of the host wall at the point of contact by a virus-packaged enzyme(s) (Figure 6). The chloroviruses encode several proteins involved in polysaccharide degradation that may be involved in degrading the cell wall (see Sect. 6.3.1). Following host cell wall degradation, the viral internal membrane presumably fuses with the host membrane, resulting in entry of the viral DNA and virion-associated proteins into the cell, leaving an empty virus capsid attached to the cell wall. This process triggers a rapid depolarization of the host membrane (probably triggered by a virus encoded potassium channel located in the virus internal membrane) (Frohns et al. 2006) and the rapid release of potassium ions from the cell (Neupartl et al. 2008); this depolarization may function to prevent infection by a second virus (Mehmel et al. 2003; Frohns et al. 2006). The rapid loss of potassium ions and associated water fluxes from the host also lowers its turgor pressure and this lowering is postulated to aid ejection of DNA from the virions into the host (Neupartl et al. 2008).

Circumstantial evidence indicates that the viral DNA and probably DNA-associated proteins quickly move to the nucleus where early transcription is detected within 5-10 min p.i. (Schuster et al. 1986). Unlike EhV-86 early gene expression (Allen et al. 2006a), the PBCV-1 early expressed genes are scattered throughout the virus genome. Shortly after infection, host chromosomal DNA begins to be degraded (Agarkova et al. 2006), presumably to aid in inhibiting host transcription and to provide the recycling of nucleotides for viral DNA replication. In this immediate-early phase of infection, the host is reprogrammed to transcribe viral RNAs. Very little is known as to how this occurs, but chromatin remodeling may be involved. PBCV-1 encodes a 119 amino acid SET domain containing protein (referred to as vSET) that di-methylates Lys27 in histone 3 (Manzur et al. 2003). vSET is packaged in the PBCV-1 virion and accumulating evidence indicates that vSET is involved in repression of host transcription following PBCV-1 infection (S. Mujtaba et al., unpublished data).

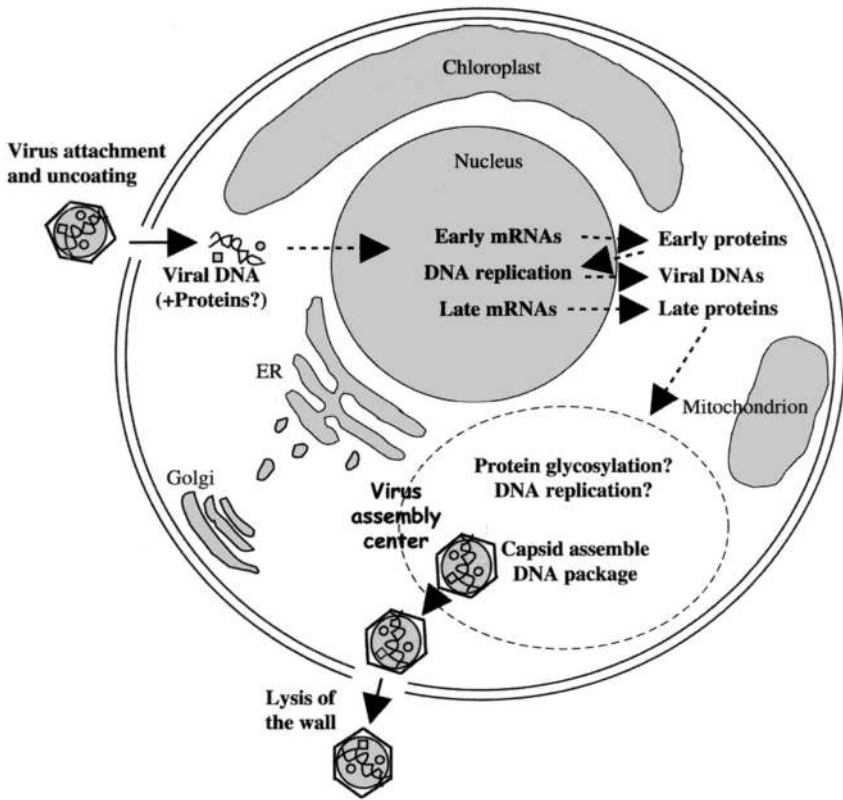


Figure 6. Proposed replication cycle of chlorella virus PBCV-1. The virus uncoats at the surface of the alga and the viral DNA, possibly with associated proteins, is assumed to move to the nucleus where early gene transcription begins within 5-10 min postinfection (p.i.). The early mRNAs are transported to the cytoplasm for translation, and the early proteins presumably return to the nucleus to initiate DNA replication, which begins 60-90 min p.i., followed by late gene transcription. Late mRNAs are transported to the cytoplasm for translation, and many of these late proteins are targeted to the cytoplasmically located virus assembly centers, where virus capsids are formed. The algal cell membrane and wall lyses and infectious PBCV-1 progeny viruses are released at 6-8 h p.i. *Solid arrows* are known events; *dotted arrows* are hypothesized events. (Reproduced with permission from Kang et al. 2005).

Viral DNA replication begins 60-90 min after infection (Van Etten et al. 1984) and is followed by transcription of late genes (Schuster et al. 1986). Ultrastructural studies of PBCV-1-infected chlorella suggest that the nuclear membrane remains intact, at least during the early stages of virus replication (Meints et al. 1986). However, a functional host nucleus is not required for virus replication since PBCV-1 can replicate, albeit poorly and with a small burst size, in UV-irradiated cells (Van Etten et al. 1986). Approximately 2-3 h postinfection (p.i.), assembly of virus capsids begins in localized regions in the cytoplasm, called virus assembly centers, which become prominent at 3-4 h p.i. (Meints et al. 1986). By 5-6 h p.i., the cytoplasm becomes filled with

infectious progeny virus particles and by 6-8 h p.i., localized lysis of the host cell releases progeny (Van Etten et al. 1983b). Approximately 1,000 particles are released per cell, of which approximately 30% are infectious.

A different lifestyle occurs in the phaeoviruses: in contrast to the lytic lifestyle of the chloroviruses, the phaeoviruses are lysogenic (Müller et al. 1998; Van Etten et al. 2002). Viruses EsV-1 and EfasV-1 only infect free-swimming, wall-less gametes of their filamentous hosts *Ectocarpus siliculosus* and *Ectocarpus fasciculatus*, respectively. Following virion attachment, the viral genomic DNA and associated protein material is released into the cytoplasm and then immediately moves to the nucleus within 5 min p.i. (Maier et al. 2002). The genomic material is then integrated into the host genome (presumably using a virally encoded integrase like the one in virus EsV-1; R.H. Meints et al., unpublished data). The viral genome is transmitted mitotically to all the cells in the developing host thallus (Braütigam et al. 1995; Delaroque et al. 1999; Müller et al. 1991). The virus genome remains latent in the vegetative filamentous cells until the formation of algal reproductive cells, either sporangia or gametangia, in the mature host. This differentiation triggers virus DNA replication in the sporangia or gametangia, followed by breakdown of the nuclear membrane, virion assembly and release of infectious virions from the cells (Wolf et al. 1998, 2000; Müller et al. 1998).

The coccolithoviruses have a different propagation strategy than either the lytic PBCV-1 or the latent EsV-1 systems, which may be similar to the lifestyle of the ancestral phycodnavirus (Allen et al. 2006c). The host alga *E. huxleyi* is covered with a calcium carbonate shell (Figure 2B) that would appear to create a physical barrier to virus adsorption. However, despite this barrier, virus adsorption is rapid and appears to be intrinsically linked to the cell cycle of the host (W.H. Wilson, unpublished data). Whereas both PBCV-1 and EsV-1 are completely dependent on the host transcriptional machinery, the coccolithoviruses are unique among the phycodnaviruses in that they have several RNA polymerase encoding genes (Wilson et al. 2005a). These genes suggest a viral replication strategy that could be either entirely or partially independent of the host nucleus. Viral transcription begins immediately after infection, but it is limited to a distinct and localized 100-kb region in the virus genome; this region contains a unique promoter element (Allen et al. 2006a, 2006d). The only genes transcribed during the Isth after infection have this element. None of these genes match anything in the databases and are referred to as ORFans (Fischer and Eisenberg 1999). Intriguingly, proteomic analysis of EhV-86 virions did not detect any transcriptional machinery packaged in mature virions, therefore a host nuclear RNA polymerase(s) is presumably responsible for this early transcription (M.J. Allen, unpublished data). Between 1 and 2 h p.i., a second transcriptional phase begins with gene expression occurring from the remainder of the genome. Since viral RNA polymerase components are expressed in this second phase, viral replication may no longer be nuclear dependent and transcription may move to the cytoplasm. Unlike the other phycodnavirus systems, where nascent virions accumulate in the cytoplasm prior to release by cell lysis, the coccolithovirus virions are released gradually in a controlled fashion. One possibility is that the virus uti-

lizes the ready-made lith export pathway to maintain this controlled and orderly release. It has also been suggested that the coccolithoviruses use the virally encoded sphingolipid metabolism pathway to manipulate the sphingolipids in the host cell wall or membrane, possibly to prevent programmed cell death and to prolong the length of infection (Han et al. 2006).

Genome Structure and Properties

By definition, all phycodnaviruses have large dsDNA genomes (Wilson et al. 2005b). These genomes range in size from 100 kb to over 550 kb with G+C contents ranging from 40% to 50% (Van Etten and Meints 1999; Dunigan et al. 2006). Complete genome sequences are available for members in three *Phycodnaviridae* genera: six chloroviruses, PBCV-1, NY-2A, AR158, MT325, FR483 and ATCV-1 (Li et al. 1997; Fitzgerald et al. 2007a, 2007b, 2007c), two phaeoviruses EsV-1 and FirrV (Delaroque et al. 2001, 2003) and the Coccolithovirus EhV-86 (Wilson et al. 2005a; Allen et al. 2006b). Partial sequence (approximately 80%) is available for a second Coccolithovirus, EhV-163 (Allen et al. 2006b). Additional phycodnavirus genomes are being sequenced, but are not yet publicly available.

There is considerable variation in genome structure among the phycodnaviruses. The PBCV-1 genome is a linear 330-kb, nonpermuted dsDNA molecule with covalently closed hairpin termini (Rohozinski et al. 1989). The termini consist of 35 nucleotide-long covalently closed hairpin loops that exist in one of two forms; the two forms are complementary when the 35-nucleotide sequences are inverted (flip-flop) (Zhang et al. 1994). Identical 2221-bp inverted repeats are adjacent to each hairpin end (Strasser et al. 1991). The remainder of the PBCV-1 genome contains primarily single-copy DNA. EsV-1 has a linear dsDNA genome with almost perfect inverted repeats at each end allowing for circularization of the genome (Delaroque et al. 2001). Indeed, prior to sequencing, EsV-1 was thought to have a circular genome (Lanka et al. 1993). It is proposed that the inverted repeats anneal with each other to form a cruciform structure that effectively circularizes the genome (Delaroque et al. 2001).

EhV-86 was originally proposed to have a linear genome (Schroeder et al. 2002). However, PCR amplification over the termini revealed a random A/T single nucleotide overhang (50% A, 50% T), suggesting the virus genome has both linear and circular phases (Wilson et al. 2005a; Allen et al. 2006a, 2006d). The detection of a DNA ligase and four endonucleases in EhV-86 hints that a linear genome may be packaged in the virions that circularize during DNA replication (Allen et al. 2006d).

Viruses typically have compact genomes for replication efficiency and the phycodnaviruses fit this pattern with approximately one gene per 900 to 1000 bp of genomic sequence (based on the predicted 366, 404, 360, 331, 335, 329, 233 and 473 coding sequences (CDS) of PBCV-1, NY-2A, AR158, MT325, FR483, ATCV-1 and EhV-86 (Table 1) (Li et al. 1997; Wilson et al. 2005a; Allen et al. 2006a; Fitzgerald et al. 2007a, 2007b, 2007c). One exception is the 336-kb EsV-1 genome that only has 231 protein encoding genes, which gives it one gene per

Table 1 Genome data of sequenced phycodnaviruses

Genus	Virus	Host	Genome size (bp)	%G+C content	ORFs	tRNAs	Nts/gene
<i>Chlorovirus</i>	PBCV-1	<i>Chlorella</i> NC64A	330,743 ^a	40.0	366	11	904
	NY-2A	<i>Chlorella</i> NC64A	368,683 ^a	40.7	404	7	913
	AR158	<i>Chlorella</i> NC64A	344,690 ^a	40.8	360	6	957
	MT325	<i>Chlorella</i> Pbi	314,335 ^a	45.3	331	10	950
	FR483	<i>Chlorella</i> Pbi	321,240 ^a	44.6	335	9	959
	ATCV-1	<i>Chlorella</i> SAG 3.83	288,047 ^a	49.4	329	11	875
	<i>Phaeovirus</i>	EsV-1	<i>Ectocarpus</i> <i>siliculosus</i>	335,593	51.7	231	0
FirrV-1		<i>Feldmannia</i> <i>irregularis</i>	191,667 ^b	156	0	1229	
<i>Coccolithovirus</i>	EhV-86	<i>Emiliania</i> <i>huxleyi</i>	407,339	40.2	472	5	863

^a Does not include the nts at the hairpin ends

^b Is not one continuous contig

roughly 1450 bp. The 366 PBCV-1 protein-encoding genes are evenly distributed on both strands and, with one exception, intergenic space is minimal. In fact, 275 ORFs are separated by fewer than 100 nucleotides. The exception is a 1788-nucleotide sequence near the middle of the genome. This DNA region, which contains many stop codons in all reading frames, encodes 11 tRNA genes.

However, some repetitive DNA occurs in the PBCV-1, EsV-1 and EhV-86 genomes. Both EsV-1 and PBCV-1 contain approximately 2-kb inverted repeats adjacent to the terminal ends (Strasser et al. 1991; Delaroque et al. 2001). In addition to the terminal repeats, tandem repeats are located throughout the EsV-1 genome and comprise approximately 12% of the total genome size (Delaroque et al. 2001). A similar proportion of the *Feldmannia* sp. virus (FsV) genome also consists of repetitive DNA (Lee et al. 1995). Intriguingly, EhV-86 has three repeat families (none of which are located at the ends of the genome); one family is postulated to act as an origin of replication (adding credence to the circular mode of replication model), another family is postulated to contain immediate early promoter elements and the last family has a large repetitive proline rich domain that may bind calcium (Allen et al. 2006d). The repetitive regions in these *Phycodnaviridae* genomes, while hindering sequencing projects, may play a role in recombination between viruses that allows genetic information to be exchanged with themselves and with their hosts (Allen et al. 2006b, 2006c).

Limited sequence information is currently available for the phycodnavirus hosts, although this situation is changing. The *Chlorella* NC64A and *E. huxleyi* genomes are currently being sequenced by the Joint Genome Institute and drafts of the two genomes will soon be available. There is evidence

that both *E. huxleyi* and EhV-86 share a putative phosphate permease gene, the large degree of difference between the genes suggesting this transfer was not recent; however, the best and only match of the virus was from the host (Wilson et al. 2005a). Host *E. huxleyi* also appears to contain numerous G+C-rich repetitive elements (B. Read, personal communication) similar to the repetitive elements in EhV-86 (Allen et al. 2006d); therefore the virus may have acquired these elements from its host.

The origin of the chlorella virus genes is a mystery. Microarrays that contain all 366 PBCV-1 genes are available and only two of the PBCV-1 genes hybridize with host chlorella DNA (G. Yanai-Balser et al., unpublished data). Furthermore, the G+C content of PBCV-1 and *Chlorella* NC64A differ significantly, 40% versus 67% (Van Etten et al. 1985). Therefore, it seems unlikely that PBCV-1 recently acquired its genes from this host.

Some of the phycodnavirus genomes have methylated bases. For example, genomes from 37 chlorella viruses contain 5-methylcytosine (5mC) in amounts varying from 0.12% to 47.5% of the total cytosine. In addition, 24 of the 37 viral DNAs contain N6-methyladenine (6 mA) in amounts varying from 1.5% to 37% of the total adenine (Van Etten et al. 1991). The methylated bases occur in specific DNA sequences; thus it is not surprising that the chlorella viruses encode multiple 5mC and 6 mA DNA methyltransferases. However, it was a surprise to discover that approximately 25% of the virus-encoded DNA methyltransferases have a companion DNA site-specific (restriction) endonuclease, some which have unique specificities (e.g., Xia et al. 1986, 1987; Nelson et al. 1998; Van Etten et al. 1991). Some of the virus encoded site-specific endonucleases are unusual in that they only cleave one strand of dsDNA, for example virus NYs-1 nickase cleaves /CC sites (Xia et al. 1988; Chan et al. 2004) and NY-2A nickase cleaves R/AG sites (Zhang et al. 1998; Chan et al. 2006). Thus, the virus-infected chlorellae are the first non-prokaryotic source of DNA site-specific endonucleases.

The phaeovirus EsV-1 genome also has low levels of methylated bases (1% and 3% of the total cytosines and adenines, respectively, are methylated) (Lanka et al. 1993; Delaroque et al. 2001). However, the EsV-1 DNA methyltransferases do not have companion site-specific endonucleases.

Coding Potential

tRNAs f IntronSf and Interns

In general, CDSs tend to be evenly distributed on both strands with minimal intergenic spaces. Exceptions to this rule include a 1788-nucleotide sequence in the middle of the PBCV-1 genome that contains a polycistronic gene encoding 11 tRNA genes (co-transcribed as a large precursor and then processed to mature tRNAs) (Dunigan et al. 2006). All the sequenced chloroviruses have a similar pattern, although the nature of the tRNAs and their genomic locations vary among the viruses (ATCV-1, 11 tRNAs, two duplicated;

MT325, ten tRNAs, one duplicated; FR483, nine tRNAs, one duplicated; NY-2A, seven tRNAs, one duplicated; AR158, six tRNAs, one duplicated) (Fitzgerald et al. 2007a, 2007b, 2007c). The coccolithovirus, EhV-86, encodes five tRNAs, four of which are clustered together (Wilson et al. 2005a). A second EhV strain (EhV-99BI) contains a sixth tRNA and also encodes a putative isoleucyl tRNA synthetase to complement the Ile-tRNA (A. Lanzen, personal communication). In contrast, the phaeovirus EsV-1 does not have any tRNA encoding genes (Delarouge et al. 2001).

Codon usage in viral proteins is often correlated with the abundance and nature of virus-encoded tRNAs (Kropinski and Sibbald 1999). However, it is interesting that there is such a large variation in the number and type of tRNAs contained within these phycodnavirus genomes. Not only does the number of tRNAs in closely related viruses (often infecting the same host) differ, but the nature of the tRNAs also vary. For example, only three tRNAs are conserved among the six chloroviruses that have been sequenced (Fitzgerald et al. 2007c).

In addition to a 13-nt intron in a PBCV-1 Tyr-tRNA gene, PBCV-1 contains two other types of introns: a self-splicing intron in a transcription factor TFIIS-like gene and a spliceosomal-processed intron in its DNA polymerase gene. Chloroviruses MT325 and FR483 lack both of these introns (Fitzgerald et al. 2007a). A spliceosomal-processed intron was also identified in a pyrimidine dimer-specific glycosylase gene in 15 chloroviruses including NY-2A and AR158 (Sun et al. 2000; Fitzgerald et al. 2007b). An intron is also present at an identical location in the ATCV-1 pyrimidine dimer-specific glycosylase gene, but this intron has little sequence identity to those from viruses NY-2A and AR158 (Fitzgerald et al. 2007c). Like PBCV, NY-2A also contains an intron in its DNA polymerase and TFIIS-like genes; in addition, NY-2A also contains another self-splicing intron in a gene of unknown function. The coccolithovirus EhV-86 contains two self-splicing introns; one in the DNA polymerase gene and one in a nucleic acid independent nucleoside triphosphatase gene (Wilson et al. 2005a).

In addition to introns, some phycodnavirus proteins contain inteins. Inteins are the protein equivalent of introns: following translation they are autocatalytically spliced to create a mature protein product. Recently, two inteins were identified in the NY-2A genome (the first to be identified in the chloroviruses): one 337 amino acid intein is in a ribonucleotide reductase large subunit protein and the other is in a putative helicase protein (384 amino acids) (Fitzgerald et al. 2007b). However, inteins may be more common in a class of phycodnaviruses that infect the raphido-phytes (Nagasaki et al. 2005). The raphidoviruses contain a 232 amino acid intein in their DNA polymerase protein, which is distantly related to an intein in the Mimivirus. This intein was present in all ten raphidoviruses examined.

Characterized Gene Products

Many PBCV-1-encoded enzymes are either the smallest or among the smallest proteins of their family. The small sizes and the finding that many

virus-encoded proteins are user-friendly have resulted in the biochemical and structural characterization of several PBCV-1 enzymes. Examples include:

1. The smallest eukaryotic ATP-dependent DNA ligase (Ho et al. 1997), which is the subject of intensive mechanistic and structural studies (Odell et al. 2003 and references cited therein).
2. The smallest type II DNA topoisomerase (Lavrukhin et al. 2000). This enzyme cleaves dsDNAs 30-50 times faster than the human type II DNA topoisomerase (Fortune et al. 2001); consequently, the enzyme from the chlorella viruses is used as a model to study the topoisomerase II DNA cleavage process (e.g., McClendon et al. 2006).
3. An RNA guanylyltransferase (Ho et al. 1996) that was the first enzyme of its type to have its crystal structure solved (Hakansson et al. 1997; Hakansson and Wigley 1998).
4. A small prolyl-4-hydroxylase that converts Pro-containing peptides into hydroxyl-Pro-containing peptides in a sequence-specific fashion (Eriksson et al. 1999).
5. An unusual thymidylate synthase, called Thy X, which usually only exists in some bacteria (Graziani et al. 2004). Unlike the traditional Thy A synthase. Thy X uses FAD for reduction (Myllykallio et al. 2002).
6. A dCMP deaminase that is unusual because the enzyme can also deaminate dCTP (Zhang et al. 2007). Cellular organisms use two enzymes that belong to different protein families to carry out these two reactions.
7. The smallest protein (94 amino acids) to form a functional K⁺ channel (Plugge et al. 2000; Kang et al. 2004). The small size of the protein lends itself to molecular dynamics simulation studies to understand how ion channels function (Tayefeh et al. 2007). An even smaller K⁺ ion channel protein (83 amino acids) was recently identified in virus ATCV-1 (Fitzgerald et al. 2007c) that forms a functional channel in *Xenopus* oocytes (S. Gazzamni et al., unpublished data). These minimalist proteins may represent precursors of contemporary proteins, but it is also possible that they are products of evolutionary optimization during viral evolution.

Recently, a serine-palmitoyltransferase (SPT) and an elongase from EhV-86 have been functionally characterized (Han et al. 2006).

Unique Genes

Novel Virus Genes

The identification of genes such as DNA polymerase, RNA polymerase, DNA ligase, helicases and topoisomerases is an excellent starting point for virus characterization. It is the genes that differ among viruses that are the source of functional diversity and they are responsible for the individual niches that each virus occupies. As noted in this chapter, members of the *Phycodnaviridae* infect a range of hosts; hence they must be highly adapted to their individual niches. In addition to containing many of the genes com-

monly found in other DNA viruses, sequencing phycodnavirus genomes have revealed genes not previously found in viruses. These gene products may provide clues as to their niche adaptation.

An example of this is the putative sphingolipid biosynthesis pathway present in EhV-86 (Wilson et al. 2005a). Sphingolipid synthesis leads to the formation of ceramide, which can affect apoptosis; thus this is an intriguing choice of a pathway for a virus to manipulate. Sphingolipids are enriched in Upid rafts and may play a crucial role during viral release (Han et al. 2006). EhV-86 contains a cluster of genes encoding sphingolipid metabolic enzymes similar to those in other organisms, from yeast to mammals (Wilson et al. 2005a). A particularly surprising example is the viral gene, *ehv050*, which encodes both subunits of the eukaryotic serine palmitoyltransferases (SPT) (Han et al. 2006). SPT is the enzyme required for the first and rate-limiting step in the pathway and catalyses the condensation of serine with palmitoyl-CoA (Hanada and Nishijima 2003). The novel fusion protein encoded by EhV-86 is not only active, it has a preference for myristoyl-CoA (Han et al. 2006). The relevance of this is unclear, but it is likely to be fundamental to the infection strategy of the virus.

The chloroviruses are also unusual because they often encode enzymes involved in sugar metabolism. Three PBCV-1 encoded enzymes glutamine: fructose-6-phosphate aminotransferase, UDP-glucose dehydrogenase (UDP-GlcDH), and hyaluronan synthase (HAS) are involved in the synthesis of hyaluronan (hyaluronic acid), a linear polysaccharide composed of alternating β -1,4-glucuronic acid and β -1,3-N-acetylglucosamine residues (DeAngelis et al. 1997; Landstein et al. 1998). All three genes are transcribed early in PBCV-1 infection and hyaluronan accumulates on the external surface of the infected chlorella cells (Graves et al. 1999).

However, not all chloroviruses have a *has* gene. Surprisingly, many chloroviruses that lack a *has* gene have a gene encoding a functional chitin synthase (CHS). Furthermore, cells infected with these viruses produce chitin fibers on their external surface (Kawasaki et al. 2002). Chitin, an insoluble linear homopolymer of P-1,2-linked N-acetyl-glucosamine residues, is a common component of insect exoskeletons, shells of crustaceans, and fungal cell walls (Muzzarelli et al. 1986). Some chloroviruses contain both *has* and *chs* genes and form both hyaluronan and chitin on the surface of their infected cells (Kawasaki et al. 2002; Yamada and Kawasaki 2005). Finally, a few chloroviruses appear to lack both genes because no extracellular polysaccharides are formed on the host surface of cells infected with these viruses (Graves et al. 1999). The fact that many chloroviruses encode enzymes involved in these energy-demanding extracellular polysaccharide biosynthetic pathways suggests that the polysaccharides are important in the virus life cycles. However, at present this function is unknown.

Two PBCV-1-encoded enzymes, GDP-D-mannose dehydratase (GMD) and fucose synthase, comprise a highly conserved, three-step pathway that converts GDP-D-mannose to GDP-L-fucose (Tonetti et al. 2003). However, the PBCV-1 GMD not only has the predicted dehydratase activity, it has a reductase activity that produces GDP-D-rhamnose. Both fucose and rhamnose

are present in the glycans attached to PBCV-1 major capsid protein. Interestingly, another chlorella virus, ATCV-1, encodes a GMD that only has the dehydratase activity (Fruscione et al. 2008).

The chlorella viruses degrade the host cell wall during infection and also during virus release. Therefore, it is not surprising that the viruses encode several enzymes that may be involved in these processes (see Yamata et al. 2006 for details). For example, PBCV-1 encodes two chitinases, a chitosanase, a β -1-3 glucanase and an enzyme that cleaves polymers of either β - or α -1,4-linked glucuronic acids (Sugimoto et al. 2004). Enzymes packaged in the virion and involved in virus infection are typically expressed late in the infection cycle and one of the chitinases and the chitosanase meet this requirement and are packaged in the PBCV-1 virion. However, the composition of the host chlorella cell walls and the enzymatic digestion of the walls during infection are still under active investigation.

PBCV-1 encodes four enzymes involved in polyamine biosynthesis: ornithine decarboxylase (ODC), homospermidine synthase, agmatine iminohydrolase, and N-carbamoylputrescine amidohydrolase (Baumann et al. 2007; Kaiser et al. 1999; Morehead et al. 2002). ODC catalyzes the decarboxylation of ornithine to putrescine, which is the first and the rate-limiting enzymatic step in the polyamine biosynthetic pathway. The PBCV-1-encoded ODC is the smallest characterized ODC (Morehead et al. 2002). The PBCV-1 enzyme is also interesting because it decarboxylates arginine better than ornithine (Shah et al. 2004). These four enzymes form a complete polyamine biosynthetic pathway that allows the formation of homospermidine through the precursors arginine, agmatine, N-carbamoylputrescine and putrescine (Baumann et al. 2007). However, the relevance of this pathway to virus replication is unclear. Although polyamines are present in large amounts in the capsids of some viruses, that is not true for PBCV-1 (Kaiser et al. 1999).

As more phycodnavirus sequences become available, we predict that the number of unique and interesting gene pathways found in these viruses will increase. Each of the viruses sequenced to date appears to have its own quirks; this pattern is likely to be repeated as more diverse phycodnaviruses are studied. For example, EsV-1 contains at least six putative hybrid histidine kinases (two-component systems which form part of a stimulus-responsive transduction pathway) that are wide-spread in archaea and bacteria, but have not previously been found in viruses (Delaroque et al. 2000, 2001). The relevance of these genes to EsV-1 infection is unknown, but is likely to be fundamental to successful infection.

Database ORFan Genes (Unknown Genes)

The wealth of genetic potential contained within the phycodnaviruses is perhaps best represented not by the unique proteins encoded by the viruses, but by the genes encoding proteins that do not have any matches in GenBank. For example, 86% of the EhV-86 gene products lack GeneBank homologs (Wilson et al. 2005a). These genes/CDSs/ORFs are commonly referred to as database ORFans (Fischer and Eisenberg 1999). Indeed, this is exemplified by a 100-kb re-

gion in EhV-86, which contains approximately 150 genes, none of which have a known function (Allen et al. 2006c). Half of these genes are associated with a unique promoter element that drives their expression, but only during the earliest stages of infection (Allen et al. 2006a, 2006d). Thus these genes encode proteins that undoubtedly play a crucial and integral role during virus infection.

Core Genes and *Phycodnaviridae* Evolution

Inter-genus Variation

There is accumulating evidence that the large dsDNA viruses referred to as nuclear- cytoplasmic large DNA viruses (NCLDV) (*Phycodnaviridae*, *Poxviridae*, *Mimiviridae*, *Asfarviridae*, and *Iridoviridae*) shared a common ancestor (Iyer et al. 2001, 2006; Raoult et al. 2004). Genome-wide bioinformatic surveys have suggested an ancestral virus genome composed of at least 50 genes that have subsequently been retained or discarded in a distinctive evolutionary traceable pattern in these highly divergent virus families (Iyer et al. 2001). Nine genes are present in all NCLDV members (known as group I genes), and 22 additional genes are present in at least three of the five families (group II and III genes). Lineage-specific gene loss and gain within NCLDV families obviously has led to the highly diverse properties of present day viruses.

High evolutionary rates, horizontal gene transfers and nonorthologous gene displacements make accurate phylogenetic resolution difficult over great periods of time, especially when using single-gene phylogenetic trees (Filee et al. 2003). Consequently, studies on whole genomes are becoming more popular when constructing evolutionary relationships between virus families (Iyer et al. 2001; Allen et al. 2006c). In a comparison of PBCV-1, EsV-1 and EhV-86, only 14 genes are common in all three viruses, indicating a large amount of inter-genus genomic variation between *Phycodnaviridae* genera (Allen et al. 2006c). A phylogenetic tree was constructed by concatenating six of the common gene products; the results indicate that the phycodnaviruses form a distinct clade from the other large DNA viruses, yet the tree fails to provide adequate resolution of the genera within the *Phycodnaviridae* (Figure 7). However, the presence of genes encoding six RNA polymerase subunits in the EhV-86 genome (many of which are found in the other NCLDV virus families) clearly separates EhV-86 from PBCV-1 and EsV-1. This finding suggests that the ancestral *Phycodnaviridae* lineage diverged with one branch giving rise to EhV-86 and the second branch giving rise to the PBCV-1 and EsV-1 lineages. The change in lifestyle represented by this loss of RNA polymerase function (i.e., from nuclear independence to nuclear dependent transcription) probably contributes to the high diversity among present day genera in the *Phycodnaviridae*. Since ancestral NCLDVs contained RNA polymerase function, it is likely that of all the phycodnaviruses sequenced so far, EhV-86 represents the virus with the lifestyle most similar to the ancestral virus (Allen et al. 2006c). As more phycodnavirus genomic sequences become available, we may be able to elucidate the complex history of phycodnavirus evolution.

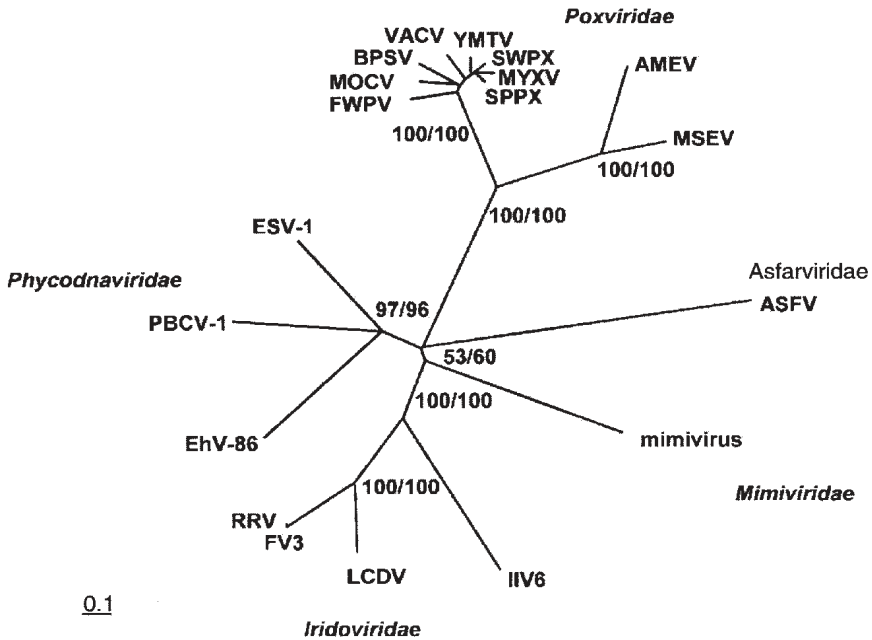


Figure 7. Phylogenetic inference tree based on a distance matrix algorithm between concatenated conserved domains from A18-like helicase, D6R-like helicase, A32-like ATPase, DNA polymerase, thio-oxidoreductase from members of the NCLDV group (Neighbor, in PHY-LIP version 3.6b). Numbers at nodes indicate bootstrap values retrieved from 100 replicates for both the neighbor-joining and parsimony analyses. The bar depicts one base substitution per ten amino acids. (Diagram adapted from Allen et al. 2006d). Viruses included are African swine fever virus (ASFV), *Amsacta moorei* entomopoxvirus (AMEV), *Melanoplus sanguinipes* entomopoxvirus (MSEV), bovine papular stomatitis virus (BPSV), fowlpox virus (FWPV), sheeppox virus (SPPX), swinepox virus (SWPX), vaccinia virus (VACV), *Molluscum contagiosum* virus (MOCV), myxoma virus (MYXV), Yaba monkey tumor virus (YMTV), *Paramecium bursaria* chlorella virus 1 (PBCV-1), *Ectocarpus siliculosus* virus 1 (ESV-1), *Emiliania huxleyi* virus 86 (EhV-86), frog virus 3 (FV3), invertebrate iridescent virus 6 (IIV6), Regina ranavirus (RRV), lymphocystis disease virus 1 (LCDV) and mimivirus.

Intra-genus Variation

With little sequence and genomic data available from the majority of the phycodnaviruses identified so far, it is difficult to assess intra-genus variation. Currently, sequencing efforts have focused on members in just three families: the chloroviruses, phaeoviruses and coccolithoviruses. Therefore, we are just beginning to appreciate the genetic diversity that exists among these genera. Initial results indicate that the huge degree of inter-genus variation can, at least, be matched by a large intra-genus variation.

Chloroviruses

The six sequenced chlorella viruses provide the best opportunity to study intra-genus variation within the *Phycodnaviridae*. The chlorella viruses are grouped by the host they infect: viruses PBCV-1, NY-2A and AR158 infect *Chlorella* NC64A (Fitzgerald et al. 2007b), MT325 and FR483 infect *Chlorella* Pbi (Fitzgerald et al. 2007a), and ATCV-1 infects *Chlorella* SAG 3.83 (Fitzgerald et al. 2007c). Approximately 80% of the genes are found in all six chloroviruses, suggesting that the majority of the genes are essential for successful viral infection of chlorella. As expected, viruses infecting the same host are most similar; the average protein amino acid identity between PBCV-1 and NY-2A or AR158 homologs is 73%, with 87% identity between NY-2A and AR158 homologs. PBCV-1 and MT325 or FR483 homologs have 50% amino acid identity; 86% identity exists between MT325 and FR483. PBCV-1 and ATCV-1 have 49% amino acid identity between homologs.

The amino acid differences between homologous proteins can be exploited to aid in understanding how certain proteins function. For example, electrophysiological experiments have been conducted with the 94 amino acid PBCV-1 encoded potassium ion channel protein (called Kcv) in *Xenopus* oocytes. Kcv-like proteins were cloned and sequenced from 40 additional chlorella viruses; 16 amino acid substitutions occurred in 94 of the amino acids, producing six new Kcv-like proteins that formed functional potassium selective channels in *Xenopus* oocytes. However, the biophysical properties of some of the new Kcv channels differed from PBCV-1 Kcv. The amino acid changes, together with the different electrophysiological properties, observed in the six Kcv-like channels were used to guide site-directed amino acid substitutions, either singularly or in combination, to identify key residues that conferred specific properties to Kcv (Kang et al. 2004; Gazzarrini et al. 2004).

Each of the six sequenced chloroviruses contains genes that encode unique proteins. For example, ATCV-1 is unique among these viruses in containing genes encoding dTDP-D-glucose 4,6 dehydratase, ribonucleotide-triphosphate reductase, and mucin-desulphatating sulphatase; MT325 encodes a functional aquaglyceroporin (Gazzarrini et al. 2006), FR483 an alkyi sulphatase and a functional potassium ion transporter (M. Kang et al., unpublished data); NY-2A an ubiquitin; AR158 a calcium transporting ATPase; and a functional Cu/Zn superoxide dismutase (M. Kang et al., unpublished data) is unique to PBCV-1.

Using the gene order of PBCV-1 as the model, there is a high degree of gene colinearity between the three viruses, PBCV-1, NY-2A and AR158 that infect *Chlorella* NC64A. Unlike the co-linearity between the three NC64A viruses, PBCV-1 has only slight co-linearity with the two viruses, MT325 and FR483 that infect *Chlorella* Pbi and virus ATCV-1 that infects *Chlorella* SAG 3.83 (L.A. Fitzgerald et al., unpublished data).

Not all PBCV-1 genes are required for virus replication in the laboratory. For example, four spontaneously derived PBCV-1 mutants were isolated that contain 27- to 37-kb deletions at the left end of the 330-kb genome (Landstein et al. 1995). Two of these mutants have deletions beginning at nucleotide coordinates 4.9 or 16 kb and ending at 42 kb. In total, these two deleted regions, which probably resulted from recombination, encode 28 putative proteins. The other two mutants, which probably arose from nonhomologous recombination, lack the entire left terminal 37-kb of the PBCV-1 genome, including the 2.2-kb terminal inverted-repeat region. The deleted left terminus was replaced by the transposition of an inverted 7.7- or 18.5-kb copy from the right end of the PBCV-1 genome. These regions encode 26 single-copy ORFs, of which 23 are common to those deleted in the first two mutant viruses. Taken together, approximately 40 kb of single-copy DNA encoding 31 ORFs at the left end of the genome, or 12% of the PBCV-1 genome, is unnecessary for PBCV-1 replication in the laboratory. However, replication of the PBCV-1 deletion mutants is attenuated; i.e., their burst sizes are about half of wild-type virus.

The deletion mutants also indicate that the size of the inverted terminal repeats in PBCV-1 can vary. This conclusion is consistent with the finding that the size and sequence of the inverted repeat region is not conserved among chlorella viruses (Strasser et al. 1991; Yamada and Higashiyama 1993). This lack of conservation is somewhat surprising because one predicts that the DNA termini might be essential for either virus DNA replication and/or DNA packaging. These results also indicate that the virus DNA packaging machinery tolerates significant differences in genome size; for example the largest deletion in PBCV-1 creates a genome of approximately 302 kb, whereas chlorella virus NY-2A has a genome of roughly 370 kb. Similar large deletions were reported in the left terminus of the chlorella virus CVK1 genome (Songsri et al. 1995).

Large insertions also occur in the chlorella viruses. Comparison of PBCV-1 and CVK2 genomes revealed that an approximately 15 kb region in the PBCV-1 left terminal region is absent in the CVK2 genome. However, CVK2 contains a 22.2-kb insert in this region that contains five gene copies of a homolog of PBCV-1 glyco-protein Vp260; this 22.2-kb sequence is absent in the PBCV-1 genome (Chuchird et al. 2002; Nishida et al. 1999).

The sizes and locations of the deletions and transpositions in the chlorella viruses resemble poxviruses (Turner and Moyer 1990) and ASFV (Blasco et al. 1989) deletion mutants. Like PBCV-1, poxviruses and ASFV genomes have inverted terminal repeats and covalently closed hairpin ends. Models to explain the generation of deletions and deletion/transpositions in the poxvirus genomes (Shchelkunov and Totmenin 1995; Turner and Moyer 1990) may be relevant to the chlorella viruses.

Several other observations reflect the diversity of chlorella virus genomes.

1. The NC64A virus NY-2A genome is roughly 40 kb larger than the PBCV-1 330- kb genome and contains approximately 30 more genes (Fitzgerald et al. 2007b).
2. Southern hybridization and DNA sequence analyses indicate that not all PBCV- 1 genes exist in all of the NC64A virus isolates. Also, an extra ORF

is often inserted between co-linear genes. The insertion of extra genetic elements, referred to as morons (for more DNA), between adjacent genes also occurs in related lambda phages (Hendrix et al. 2000).

3. Sequence analyses of a gene (*pdg*) encoding a UV-specific DNA repair enzyme from 42 NC64A viruses revealed that 15 of them contain a 98-nucleotide spliceosomal-processed intron that is 100% conserved; four other viruses contain an identically positioned 81-nucleotide intron that is nearly 100% conserved (Sun et al. 2000). In contrast, the nucleotides in the *pdg* coding regions (exons) from the intron-containing viruses are 84%-100% identical. The 100% identity of the 98-nucleotide intron sequence in 15 viruses and the near 100% identity of an 81-nucleotide intron sequence in another four viruses imply that either the intron was acquired recently or that there is strong selective pressure to maintain the DNA sequence of the intron once it is in the *pdg* gene. However, the abilities of intron-containing and intron-lacking viruses to repair UV-damaged DNA in the dark were indistinguishable (Sun et al. 2000). These findings contradict the dogma that intron sequences are more variable than exon sequences.
4. Yamada and his colleagues (Nishida et al. 1998; Yamada et al. 1994) reported that 8% of the NC64A viruses isolated in Japan contain a self-splicing group I intron. This intron is inserted in the gene encoding either a transcriptional elongation factor TFIIS (~60% of the viruses) or an unidentified ORF encoding a 14.2-kDa polypeptide (~40% of the viruses); however, in a few viruses the intron is in the major capsid protein gene. Yamada et al. (1994) suggested that the self-splicing intron might function as a mobile genetic element; e.g., transferring genes between algae and protists.

To summarize, considerable variation occurs in the chlorovirus genomes and the total number of genes in the chlorovirus gene pool exceeds that of a single isolate. The different sizes of the chlorovirus genomes as well as the large deletions and insertions, suggest that dynamic and frequent rearrangements of virus genomes occur in natural environments. The fact that the left end of the chlorella virus genome is tolerant to deletions/insertions/rearrangements suggests that a recombinational hotspot in this region allows viruses to exchange genes among themselves and possibly with their host(s).

Coccolithoviruses

In addition to the fully sequenced English Channel Coccolithovirus, EhV-86, there is good sequence coverage (>80%) of a second Norwegian virus, Eh V-163 (Allen et al. 2006b). Both strains are capable of infecting many, but not all, of the same *E. huxleyi* strains and they have nearly identical sized genomes (Allen et al. 2007). The sequence of a third strain, another Norwegian strain closely related to EhV-163, is also near completion. Sequencing the coccolithoviruses is very difficult because of the highly repetitive nature of the genomes. Direct comparison of EhV-86 and Eh V-163 genomic sequences reveal that of the 202 CDSs for which there is full sequence in both genomes, only 20 are completely identical at the nucleotide level and an additional 17

at the protein level (Allen et al. 2006b). Although these 37 conserved CDSs are distributed throughout the genome, some of them appear to be clustered together in four regions. Many of the remaining fully sequenced EhV-163 CDSs have 95%-99% identity with their EhV-86 counterparts. One CDS, *ehv142*, is highly variable (86.9% at the nucleotide level, 79.1% at the amino acid level), the function of this gene product is unknown, but it may be a KELCH-like protein. KELCH-like proteins in the poxviruses contribute to virulence, host range and reproduction; therefore this EhV-86 protein may contribute to the variation in host range displayed by these viruses (Tulman et al. 2002; Pires de Miranda et al. 2003; Kochneva et al. 2005). Since the EhV-163 genome sequence is unfinished, it is not possible to draw any firm conclusions as to which genes are absent from EhV-163, but present in EhV-86. However, a genomic rearrangement occurs in EhV-163. A putative EhV-86 phosphate permease (*ehv117*) has been partially deleted and replaced with a putative endonuclease (*ehv117A*) (Allen et al. 2006b). The significance of this replacement is unknown, but it may represent an overall like-for-like replacement because both gene products have the potential to increase the phosphate available to the virus, though by very different mechanisms. One mechanism is by increasing phosphate uptake and the other by degrading host nucleic acid.

A genome-wide microarray-based survey, used to assess the genomic variation without sequencing, has revealed a large amount of variation in genomic content among the coccolithoviruses (Allen and Wilson 2006; Allen et al. 2007). In a screen of a dozen virus isolates, 71 out of 425 CDSs tested were either absent or highly variable in at least one isolate. All core NCLDV genes identified in EhV-86 are present in all isolates tested to date. The advantage of this genomic snapshot analysis can be seen when considering the most phylogenetically and geographically distinct isolates, EhV-163 and EhV-V2. EhV-163 and EhV-V2 have at least 27 and 28 missing or highly variable CDSs, respectively, in comparison with EhV-86 (26 common to both strains). The largest variation occurred between isolates obtained from the same geographic location, but separated by time of sampling (Schroeder et al. 2002), suggesting that the coccolithoviruses are excellent examples of the classic evolutionary rule of thumb that variation within populations is often greater than variation between populations. Despite the large degree of variation in genome content, the overall genome size is similar between coccolithoviruses, suggesting that physical genome size, in addition to genome content, may be crucial to the virus (Allen et al. 2007). Whether this is due to genomic packaging, replication or some other unknown process is not known.

Phaeoviruses

A comparison of EsV-1 (231 genes, 335 kbp) and FiiV-1 (156 genes, 231 kbp) genomes revealed that 93 FiiV-1 genes are related to EsV-genes (Delaunoy et al. 2003). The most highly conserved genes (>50% amino acid identity) predictably include the core NCLDV genes. The majority of the virus-encoded proteins have 31%-50% amino acid identity. However, the gene order is remarkably different between the two viral genomes, suggesting a

high rate of recombination and divergence since their last common ancestor. Whereas the chloroviruses and coccolithoviruses (discussed in Sects. 7.2.1 and 7.2.2) only infect one algal species, phaeoviruses often infect two distinct, but taxonomically similar, filamentous brown algae, *Ectocarpus* and *Feldmannia* (Delaroque et al. 2003). Despite the closely related host organisms and similar life styles, their genomes are remarkably different. Partial genomic sequence is also available for another *Feldmannia* virus, FsV, and the gene order is similar to that of FiiV-1. Of particular interest are large sections of the EsV genome that have no FiiV-1 homologs; presumably these sections of the genome have either been lost in the FiiV-1 lineage or acquired by the EsV-1 lineage (Delaroque et al. 2001, 2003). It is possible that the genes obtained in this region are essential for successful infection of *Ectocarpus* hosts, but are not needed for *Feldmannia* infection. However, these viruses have almost indistinguishable lysogenic replication cycles and the extra genomic regions in EsV-1 may be predominantly redundant (Delaroque et al. 2003).

Summary

The *Phycodnaviridae* is an ancient, genetically diverse, yet morphologically similar family of large icosahedral viruses that infect eukaryotic algae; they have dsDNA genomes ranging from 180 to 560 kb. Currently, the *Phycodnaviridae* consist of six genera named after the hosts they infect (*Chlorovirus*, *Coccolithovirus*, *Prasinovirus*, *Prymnesiovirus*, *Phaeovirus* and *Raphidovirus*). Their hosts, eukaryotic algae, are an incredibly diverse group of organisms that represent at least five distinct evolutionary lineages. They are ubiquitous in marine, freshwater and terrestrial habitats, and the number of algal species may be as high as several million. Their role in global carbon and nutrient cycling make O₂-evolving eukaryotic algae arguably the most important microorganisms for maintaining the status quo of life on the planet. It is likely that viruses infect all eukaryotic algae and it is reasonable to assume that many of these viruses will be assigned to the *Phycodnaviridae*. Therefore, it is perhaps surprising that only a few hundred (at most) phycodnaviruses have been isolated, less than a dozen have been sequenced and only one (PBCV-1) has been studied in any depth. Thus it is obvious that a huge gap exists in our knowledge and understanding of the *Phycodnaviridae*.

The *Phycodnaviridae* clearly group into a single family. Their common evolutionary origin is reflected not only in their appearance (despite their range in sizes, these viruses are structurally and morphologically similar) but also in their genomic content. As with the other NCLDV viruses (*Poxviridae*, *Iridoviridae*, *Mimiviridae*, and *Asfarviridae*), their common evolutionary origin is revealed by the so-called core genes, which are responsible for essential viral functions such as DNA replication, RNA transcription and virion assembly; these genes have been conserved since their ancient last common ancestor. Yet the enormous divergence that has occurred since that ancestral virus graced the world has created a truly fascinating group of viruses that will enthral researchers for many years to come. We have hardly scratched the sur-

face in studies on the *Phycodnaviridae*, yet it is already obvious that the highly diverse nature of the host algae is also reflected in the diversity of the phycodnaviruses. As the host organisms have diverged to occupy their own specific niches, these viruses have responded to the challenge and evolved to survive in the wide range of environments they now find themselves. This is reflected in the huge degree of variation among the viruses. So far, each phycodnavirus genera appear to be unique in lifestyle, with each virus having its own peculiarities and novelties. Nearly everything that can vary, does vary within the *Phycodnaviridae*, from genome size, structure (circular or linear) and content, to propagation strategy and life style. Variety is the spice of life, which makes the *Phycodnaviridae* the spiciest of the viruses. As researchers recognize the scientific and commercial worth of studying these viruses, we predict they will not be regarded as lesser known for very long.

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